Longitudinal Study of Recurrent Metastatic Melanoma Cell Lines Underscores the Individuality of Cancer Biology

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Recurrent metastatic melanoma provides a unique opportunity to analyze disease evolution in metastatic cancer. Here, we followed up eight patients with an unusually prolonged history of metastatic melanoma, who developed a total of 26 recurrences over several years. Cell lines derived from each metastasis were analyzed by comparative genomic hybridization and global transcript analysis. We observed that conserved, patient-specific characteristics remain stable in recurrent metastatic melanoma even after years and several recurrences. Differences among individual patients exceeded within-patient lesion variability, both at the DNA copy number ($P<0.001$) and RNA gene expression level ($P<0.001$). Conserved patient-specific traits included expression of several cancer/testis antigens and the *c-kit* proto-oncogene throughout multiple recurrences. Interestingly, subsequent recurrences of different patients did not display consistent or convergent changes toward a more aggressive disease phenotype. Finally, sequential recurrences of the same patient did not descend progressively from each other, as irreversible mutations such as homozygous deletions were frequently not inherited from previous metastases. This study suggests that the late evolution of metastatic melanoma, which markedly turns an indolent disease into a lethal phase, is prone to preserve case-specific traits over multiple recurrences and occurs through a series of random events that do not follow a consistent stepwise process.

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Abbreviations: aCGH, array comparative genomic hybridization; ANOVA, analysis of variance; CAN, copy number alteration; CN, copy number; FDR, false discovery rate; GX, gene expression; MDS, multidimensional scaling; RM ANOVA, repeated measures analysis of variance

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INTRODUCTION

Cancer progression is usually studied cross-sectionally, comparing lesions obtained from different patients, excised at various stages. By combining these snapshots, the natural history of the disease can be indirectly reconstructed. In contrast, the preferable longitudinal analysis of sequential lesions in the same patients is usually not feasible, especially difficult to perform in rapidly progressing cancers, such as melanoma, and particularly challenging when analyzing disease progression in metastases (Bonsing *et al.*, 1993; Kuukasjarvi *et al.*, 1997; Navin *et al.*, 2011).

However, the limited number of such longitudinal studies leaves several questions open. First, cross-sectional studies do not allow an estimate of the extent to which patient-specific traits remain stable over time. Therefore, it is difficult to assess the stability of such patient-specific traits over time, which is a question of basic importance in personalized cancer therapy (Gupta *et al.*, 2009; Harbst *et al.*, 2010; Navin *et al.*, 2010).

In addition, with cross-sectional analyses, it is impossible to test whether late disease development follows a pattern of sequential somatic microevolution or whether subsequent metastases represent individual buddings from a stable set of cancer progenitors, creating independently established new metastatic lesions (Wang *et al.*, 2006; Sabatino *et al.*, 2008).

Finally, it is difficult to quantify whether sequential steps are involved in late-stage progression, and to estimate whether consistent changes are required for the late progression of melanoma from a metastatic phase that progresses slowly to a rapid evolution in the declining phase of one patient's life.

By studying longitudinally several recurrent melanoma metastases of a rare collection of eight individuals who developed multiple recurrences over a period of years (see Supplementary Table S1 online), we sought a better understanding of the above questions. This study is a follow-up from a previous longitudinal study of a single case (Wang *et al.*, 2006; Sabatino *et al.*, 2008) focusing on traits remaining stable and changes repeated consistently among multiple developing recurrent metastases of several melanoma patients. To our best knowledge, these questions have not yet been analyzed by others.

RESULTS

Long-term metastatic melanoma is consistent with canonical melanoma genomics

As the cases with multiple recurrent metastases studied here differ behaviorally from classic metastatic melanoma owing to their unusually protracted course, we first evaluated whether the cell lines derived from these unusual cases would differ markedly from typical cases of melanoma, as published by others.

Array comparative genomic hybridization (aCGH) confirmed that the chromosomal distribution of copy number (CN) alterations (CNAs) prominently observed here are in line with previous observations (Figure 1a) (Thompson *et al.*, 1995; Roschke *et al.*, 2003; Jonsson *et al.*, 2007; Spivey *et al.*, 2012). In addition, at the individual gene level, most genes were affected by CN gains and losses in accordance with others' reports (Okamoto *et al.*, 1999; Pirker *et al.*, 2003; Grafstrom *et al.*, 2005; Jonsson *et al.*, 2007; Shi *et al.*, 2012) (Figure 1b, see full data set in Supplementary Table S2 online).

Finally, similar to others' reports, we also found that a correlation between CN and gene expression (GX) data are present but limited in advanced cancer (Sabatino *et al*, 2008; Spivey *et al*, 2012). Among 4,340 genes eligible for analysis, 2,766 correlated weakly (Pearson's correlation $R<0.3$, $P<0.05$, false discovery rate (FDR) 0.05) and 272 correlated strongly ($R<0.5$, $P<0.05$, FDR 0.01) in CN and GX (see Supplementary Figure S1 online).

Taken together, this data set was representative of typical characteristics of metastatic melanoma genomics, as reported in the literature (Thompson *et al.*, 1995; Roschke *et al.*, 2003; Jonsson *et al.*, 2007; Sabatino *et al.*, 2008; ; Spivey *et al.*, 2012).

Advanced melanoma retains case-specific fingerprints after years of disease progression

Following a rare case of metastatic melanoma that recurred several times over a decade, we previously observed that in spite of the stochastic and selective forces affecting its genome, stable characteristics prevailed to the point that recurrent lesions derived from this patient clustered away from heterologous randomly collected cases (Wang *et al.*, 2006; Sabatino *et al.*, 2008). This patient-specific stability, if shared by other cases of advanced melanoma, could have fundamental implications for personalized cancer therapy. Thus, in this study, we first analyzed whether the previous observations could be generalized to a larger set of patients.

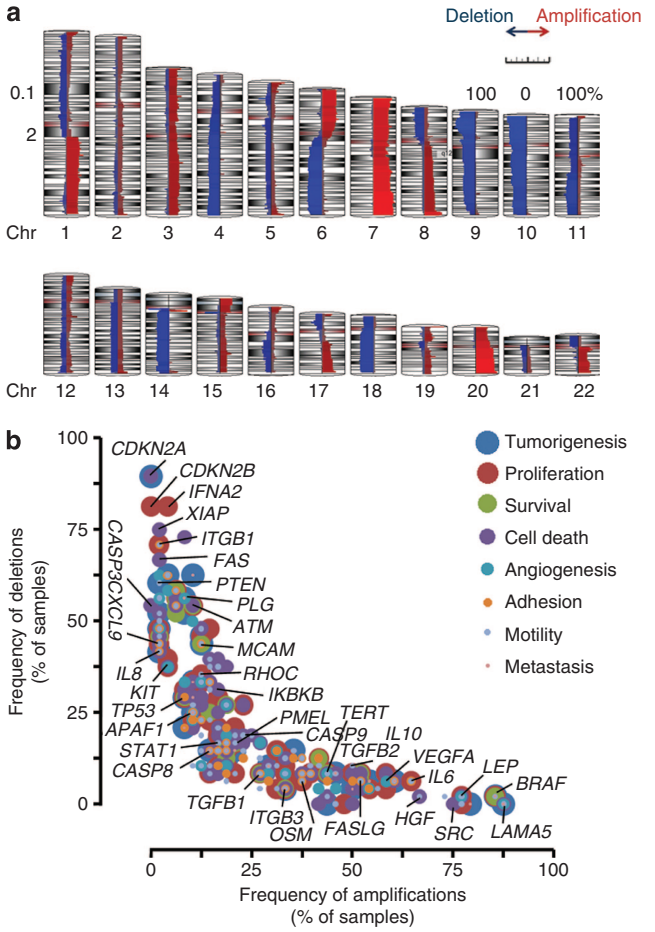

Figure 1. Description and basic characterization of the analyzed sample set by integrated copy number and gene expression analysis. (**a**) Frequency and spatial distribution of autosomal copy number (CN) aberrations in the analyzed melanoma sample set. (**b**) Combined distribution analysis of CN gains and losses affecting key melanoma genes, and also their distribution between various disease-related biological functions, as defined by the Ingenuity Pathway Analysis database. Selected key melanoma genes are labeled with their respective HUGO gene symbols.

First we compared CNA and GX patterns on a global genomic scale among cell lines from the eight patients with multiple recurrences. Multidimensional scaling (MDS), a computational method enabling visualization of sample relatedness within large-scale genetic data, demonstrated that even after years recurrent metastases of a given patient remained closely related, keeping clear distance from others' metastases (Figure 2a and c). By comparing all metastases in all possible pairs (325 pairs total), we found that MDS distances between subsequent metastases (estimates of sample relatedness) of the same patient were significantly shorter than those between metastases of different patients (Figure 2b and d). This finding held true whether CNA or GX data were compared.

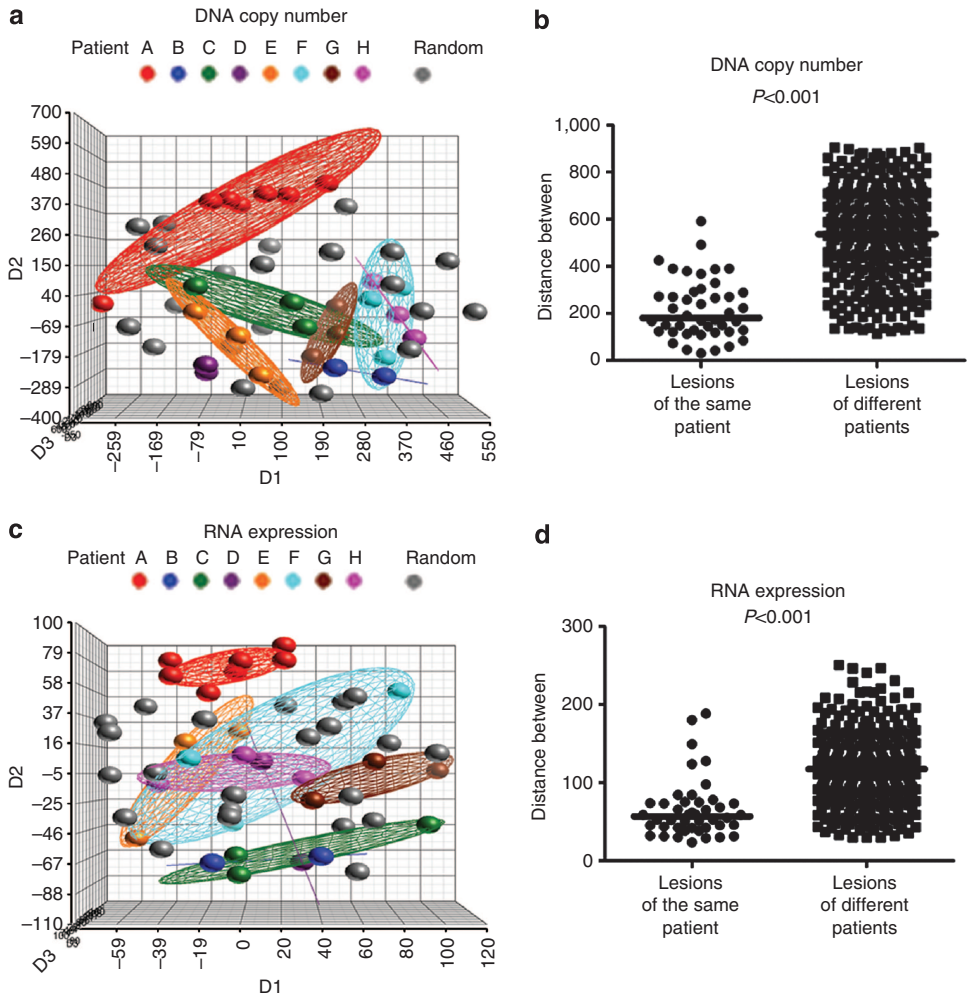

Figure 2. Comparison of the relative weights of within- versus between-patient differences in metastatic melanoma. (**a**)The whole complexity of DNA copy number data reduced to three dimensions (D1–3) by multidimensional scaling (MDS). Metastases are symbolized by spheres. (**A**–**H**) Recurrent metastases belonging to the same patient are color-coded; non-recurrent, random metastasis samples, serving as controls, are gray. (**b**) Distribution of MDS plot distances between individual metastases representing the magnitude of actual genomic differences. Statistical comparisons of MDS distances (~genomic differences) between recurrent metastases belonging to the same versus different patients are shown. *P*-values given are derived from a standard *t*-test considering all possible recurrent metastasis pairs from the sample set. Panels **c** and **d** display similar information on whole-genome RNA expression data.

Stable patient-specific traits include genes of relevance to melanoma biology

We next searched for genomic aberrations typically specific to a given patient. We found that stable case-specific CNAs occurred in chromosomes 1, 5, 13, and 19 (Figure 3a; one-way analysis of variance (ANOVA), $P<0.05$, FDR<0.001; see Supplementary Table S3 online for details). Similarly, 925 genes were found to have stable, patient-specific expression; 61 among them could be categorized functionally as melanoma-related by the Ingenuity Pathway Analysis database (Figure 3b; one-way ANOVA, $P<0.05$, FDR<0.05). The latter included several genes with known tumorigenic properties supporting autonomous proliferation (*KIT*, *MYC*, *CDK2*, *RBL2*), controlling genomic stability (*BRCA1*), apoptosis and cell survival (*TP53BP2*, *CASP8*, *TEP1*), adhesion and motility (*CDH1*, *ITGA4*), invasiveness, matrix remodeling (*MMP15*, *MMP19*), angiogenesis (*ANGPT1*, *EGF*), modulation of antitumor immunity (large clusters of major histocompatibility complex class I and II transcripts, the latter correlating with *CIITA* expression), and several melanoma antigens (*MAGE-A1*, *-A4*, *-A9*, *-B2*, *-C2*). This observation suggests that genes highly relevant to melanoma progression retain stable patient-specific expression levels over long periods of time (Figure 3b).

Notably, among all possible patient-to-patient comparisons (28 pairwise comparisons involving 8 patients), 37 genes demonstrated patient-specific expression patterns with significant differences among patients and an at least 2-fold change in >70% of all pairwise comparisons. These included *MAGE-A4*, *-B2*, *-C2*, *BAGE-2*, and *KIT* (see Supplementary Table S4 online). To further test these results, we analyzed KIT protein levels by flow cytometry in the investigated cell lines. Our analysis disclosed that, although KIT expression is frequently affected by post-transcriptional regulation, KIT protein levels remain consistent throughout multiple recurrences of individual patients, and whenever expressed they correlate well with mRNA data (Supplementary Figure S2 online). Taken together,

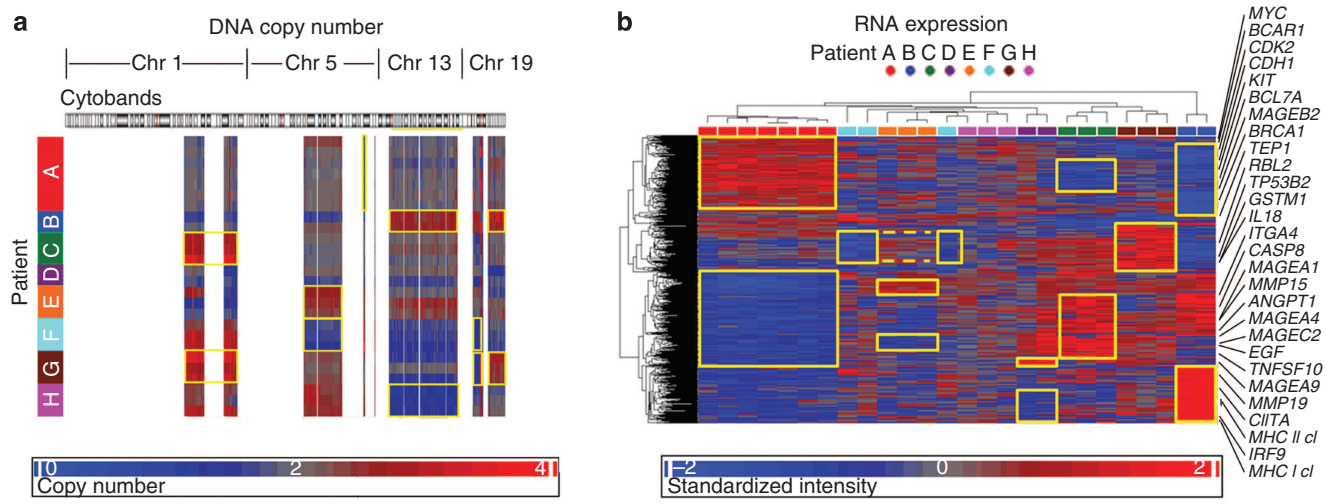

Figure 3. Identification of stable individual traits conserved in recurrent metastases of a given patient through years of ongoing disease history. (**a**) Examples are shown for stable conserved copy number traits that remain characteristic for a given case of recurrent melanoma (selected examples in yellow frames). Samples belonging to the same patient are aligned horizontally and color-coded (to the left). (**b**) Conserved gene expression patterns, characteristic for a given case, remain stable throughout multiple recurrences and are shown using a standardized heatmap (selected examples in yellow frames). Samples are aligned vertically and color-coded (top). HUGO gene symbols of selected melanoma-related genes are shown to the right.

these observations suggest that genes relevant to melanoma immunology and melanoma cell biology are expressed stably within a given patient, and may, in turn, be responsible for behavioral differences among individual cancers.

Lack of evidence for convergent evolution and consistent changes among patients over time

Next, we asked whether subsequent metastases from different patients progressively converge to reach a terminal, potentially lethal ''hyperaggressive'' status. This would imply that, on average, early (e.g., first) metastases of individual patients would be more different and more distant from each other compared with late (e.g., the last) metastases of the same individuals. MDS genomic distances demonstrate that this is not the case (Figure 4a and b), neither at the CN nor at the GX level.

To corroborate this finding, we next attempted to identify consistent CN or GX changes that might represent a recurrent theme in the transition from earlier to later metastases in a given patient. However, statistical analysis was unable to identify changes in CNAs or GX patterns that constitute consistent trends in subsequent recurrences of metastatic melanoma (two-way RM ANOVA, $P<0.05$, FDR 0.05). First, an analysis of all recurrent metastases inclusive of patient identity and lesion sequence revealed no consistent changes between subsequent metastases. Next, as patients with large numbers of recurrences dominate the analysis in such a pairwise comparison, we decreased or eliminated differences in per-patient sample sizes. To this end, we first replaced multiple synchronous metastases with a single averaged value for each parameter tested ($P<0.05$, FDR 0.05). In addition, in a separate analysis, we limited the evaluated cases to three randomly selected samples per patient ($P<0.05$, FDR 0.05). No consistent changes were found by either analysis. Next, assuming that the first and last available lesions in a given patient were most distant genetically, we restricted the analysis to these extreme pairs; but again, a pairwise analysis including patient identity failed to identify statistically significant differences ($P<0.05$, FDR 0.05). Finally, hypothesizing that the last, supposedly most advanced, fatal lesion in a given patient might be different from earlier ones, we compared the latter with the former ($P<0.05$, FDR 0.05), again without observing consistent differences. Taken together, no consistent progression patterns could be observed between subsequent metastases, either at the DNA CN or at RNA GX level, regardless of the approach used for sample selection and grouping before statistical analysis.

In line with this observation, comparison of the first metastasis from a given patient with his subsequent ones demonstrated that the latter are not necessarily drifting progressively further from the original one (Figure 4c and d). Rather, the data suggest a stochastic drift among subsequent recurrent metastases.

We also tested whether multiple cycles of phenotype switching between proposed invasive and proliferative phenotypes (Hoek *et al.*, 2008) could explain a seemingly stochastic drifting of recurrent melanoma metastases. We found that this model may provide partial explanation for our observations, as key genes of the two phenotypes were expressed in an alternating manner, and the two phenotypes seemed to change frequently back and forth through the recurrences of most (e.g., patients B, C, D, F, G), although certainly not all, patients (e.g., patients A and E, Supplementary Figure S3 online).

The fate of homozygous deletions does not support cumulative changes in the evolution of melanoma

As no stepwise evolutionary pattern could be discerned, we next asked whether recurrent metastases from the same patient descend sequentially from one another, i.e., if they acquire

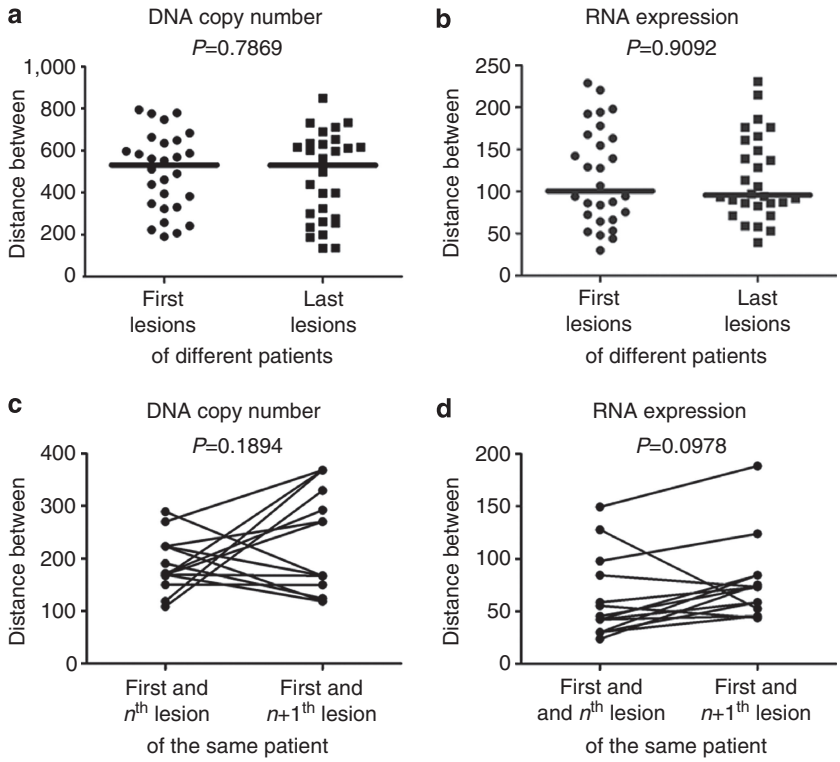

Figure 4. Testing evolutional convergence and sequential evolution in recurrent metastatic melanomas on a global scale. (**a**, **b**) Compare MDS-based distances (estimates of genomic difference) between first and last lesions of different patients experiencing multiple recurrences of melanoma. A standard *t*-test is applied to test whether late lesions are less different from each other than earlier ones, that is, whether there is convergent evolution among individual cases of metastatic melanoma. (**c**, **d**) Analyze the question whether subsequent recurrent recurrences (any *n*th and *n* + 1th lesion) would be more and more distant (~different) from the first diagnosed metastasis, implying incremental changes and thus sequential evolution of subsequent metastases.

new mutations in a cumulative manner. To this end, we followed up the fate of common *BRAF* and *NRAS* mutations (Colombino *et al.*, 2012) and homozygous deletions (−/−) in subsequent recurrent metastases. Unfortunately, *BRAF* and *NRAS* status turned out to be uninformative in this regard, because as frequently observed in melanoma all recurrent melanomas analyzed were BRAFV600E mutated and NRAS wild type throughout (not shown). Next, we analyzed the fate of homozygous deletions (−/−) that are thought to be irreversible, as no known mechanisms for structural restoration of these alterations have been described. On the basis of this, we assumed that if subsequent recurrent metastases of the same patient show reversions of homozygous deletions, they cannot sequentially descend from each other.

A total of 33 contiguous homozygous deletions were found to affect the *CDKN2A/CDKN2B* region, various IFN genes, *B2M*, major histocompatibility complex genes, etc. Out of these, 25 deletions were eligible for analysis, as they emerged in a metastasis for which there was at least a subsequent metastasis to evaluate (Figure 5b). Out of 25 eligible homozygous deletions, 15 (60%) appeared to be reverted in a given patient's disease history, suggesting that in subsequent metastases of recurrent melanoma new mutations are not acquired in a cumulative manner, and hence recurrent metastases do not descend from each other (Figure 5b).

Recurrent melanomas show hints of slower growth, but more frequent metastasis formation

In initial MDS analyses, cancer cells from patients with recurrent long-term metastatic disease were hardly discernible from those from sporadically excised, melanoma cases (Figure 2a and c). Nevertheless, we identified a set of 177 genes differentially expressed between the two phenotypes, which is a very small number compared with patient-to-patient differences, eight of which were melanoma-related. Interestingly, these genes hint to slower tumor growth (retained *CDKN1A* and *ANAPC* expression), higher sensitivity to immune- or therapy-mediated eradication (higher *FAS* but lower levels of *MGMT* expression), and higher prometastatic tendency (elevated levels of *ALCAM*; Supplementary Figure S4 online).

DISCUSSION

This study analyzes a specific time point in the natural history of cancer when advanced disease of an indolent nature turns into an aggressive and lethal stage. We studied the genetic profile of melanoma cell lines derived from sequentially excised metastases in unusual cases when the metastatic process followed a protracted course. Although the use of cell lines has significant limitations, we observed that early-passage cell lines maintain stable genetic traits *in vitro* that relate to the *in vivo* phenotype of parental tumors (Spivey

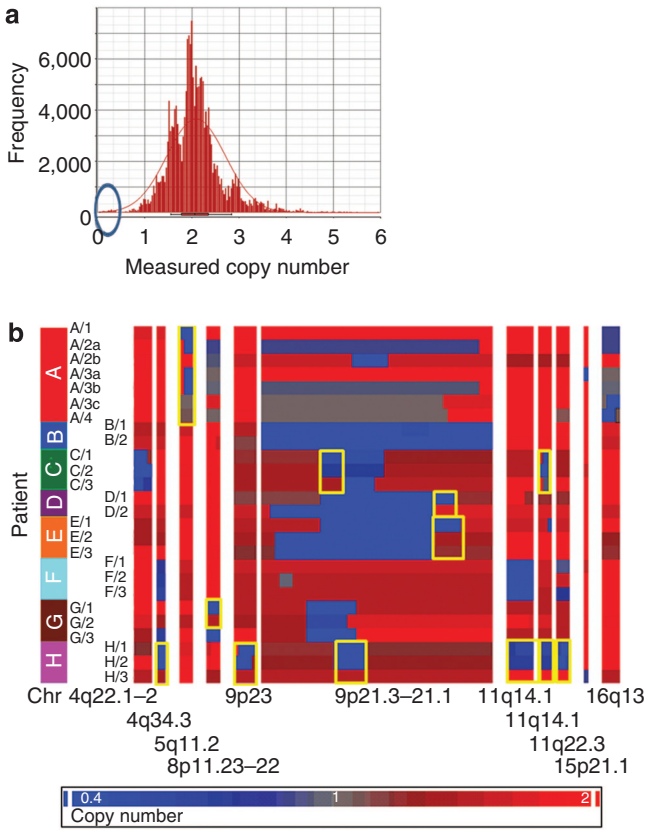

Figure 5. Follow-up analysis of the stability of homozygous deletions in evolving recurrent metastatic melanomas. (**a**) A histogram of the calculated DNA copy number values associated with every identified chromosome segment in the analyzed melanoma sample set. A blue circle marks segments accepted as homozygous deletions (−/−) considering the accuracy and statistical fidelity limits set for chromosomal segmentation. (**b**) The fate of these completely deleted segments in eight patients (A, B, etc.) experiencing several melanoma recurrences in a sequence (A/1, A/2, B/1, B/2, etc.), some of which are multiple synchronous recurrences (A/2a, A/2b, etc.). Yellow frames indicate selected chromosomal regions that, although completely lost at one time point of disease history (−/− = blue), months or years later re-emerged in a recurrence of the same case of cancer (−/+ = gray or +/+ = red).

et al., 2012). Nevertheless, our samples clearly do not equal whole tumors, and these cases may have represented a special subset of melanoma as well. First, these recurrent melanomas displayed CDK2NA, PTEN, and BRAF CN aberrations more frequently than average cases (Hodis *et al.*, 2012; Krauthammer *et al.*, 2012). In addition, all 26 metastases of the analyzed eight patients carried BRAFV600E, but displayed wild-type NRAS. Conservation of *BRAF* mutation status across metastases is in line with others' observations (Niessner *et al.*, 2013). However, this particular BRAF/NRAS pattern is typical for melanomas arising in intermittently sun-exposed areas (Colombino *et al.*, 2012), affects cell proliferation rate (Liu *et al.*, 2007), prognosis (Long *et al.*, 2011), treatment of choice, and, in this latter context, also BRAF CNs (Shi *et al.*, 2012).

Keeping these limitations in mind, our data suggest that key elements of the framework of recurrent metastatic melanomas remain stable with time; as such stability was observed in eight out of eight patients, it possibly represents the rule rather than the exception. This is a remarkable finding considering that at the same time our data also support the accepted view of late-stage cancer evolution being a highly dynamic process, also shown recently by others (Gerlinger *et al.*, 2012; Shah *et al.*, 2012) using indirect computational inference; however, this study uniquely provides direct evidence by studying serially asynchronous metastases over a long period.

Our findings suggest that individuality is maintained throughout a non-directional drift that does not follow a clearly linear progression, with each metastatic signature stemming *de novo* from a stable progenitor entity. Moreover, there was no sign of a convergent evolution in advanced late-stage melanoma toward the creation of a convergent lethal phenotype, and recurrent metastases did not seem to be each other's clonal descendants, or accumulate incremental changes, which is in line with others' recently published observations (Colombino *et al.*, 2012).

On the other hand, stable expression of cancer/testis antigens and the *c-kit* proto-oncogene across multiple recurrences of melanoma implies that late-stage melanoma is capable of displaying stable, case-specific differences directly affecting markers that determine vulnerability to next generation immunological or small-molecule biotherapy (Tyagi *et al.*, 2005; Tyagi and Mirakhur, 2009; Guo *et al.*, 2011).

It remains to be clarified to what extent these observations are attributable to the effects of clonal heterogeneity (Gerlinger *et al.*, 2012; Shah *et al.*, 2012), circulating tumor cells (Maheswaran *et al.*, 2008; Yu *et al.*, 2011) that may remain dormant for years and reset the evolutionary clock upon their reactivation, multiple events of phenotype switching (Hoek *et al.*, 2008; Eichhoff *et al.*, 2010), or persistent cancer stem cells opening multiple alternative ways to cancer evolution with each individual recurrence (La Porta, 2012; Shakhova and Sommer, 2012). Larger and more comprehensive studies involving genome-wide DNA sequencing, epigenetic and proteomic analyses, analyzing patients with average survival times, and resected whole tumors instead of cell lines are strongly warranted to clarify these questions and confirm the applicability of our findings to usual cases of advanced melanoma.

MATERIALS AND METHODS

Patients and samples

Twenty-six recurrent melanoma metastases were surgically isolated from eight patients experiencing relapse after one or more successful treatment intervention(s) with no signs of residual disease. Recurrent metastases from different tissues appeared in periods spanning 10–148 months, with 8–101 months between recurrences (see Supplementary Table S1 online for all data regarding samples, patients, treatments, and disease history). Patients received therapy and underwent surgery at the Surgery Branch of the National Cancer Institute, National Institutes of Health, Bethesda, MD, or at the Centro di Riferimento Oncologico (Italian National Cancer Institute) in Aviano, Italy. Patients were treated and samples were obtained after signing written informed consent approved by each institute's review

board, and in accordance with the Declaration of Helsinki Principles. From all lesions, stable cell cultures were established and maintained at the Department of Transfusion Medicine, Clinical Center, and National Institutes of Health for at least eight passages. Patients experiencing recurrent metastases were labeled with capital letters ''A,'' ''B,'' ''C,'' etc., their subsequent metastases were labeled as ''A/1,'' ''A/2,'' ''B/1,'' ''B/2,'' etc., whereas synchronous metastases in a given patient were labeled as ''A/1a,'' ''A/1b,'' etc. All recurrent melanoma metastases analyzed appeared after a single primary tumor. Another 22 melanoma cell lines isolated and maintained as above were expanded from melanoma patients with rapid disease course, for whom only one metastasis was available. As no extended follow-up was possible in these cases, the cell lines are considered representative of random time points in the natural course of metastatic melanoma. These cell lines were labeled with Arabic numbers as ''1,'' ''2,'' ''3,'' etc.

DNA isolation

Total genomic DNA of cell lines was isolated using the QuickGene DNA whole-blood kit S and a QuickGene-810 Nucleic Acid Isolation System (Fujifilm, Tokyo, Japan).

HLA typing

To exclude accidental cross-contamination of samples, low-resolution HLA typing was performed at the HLA Laboratory, Laboratory Services Section, Department of Transfusion Medicine, Clinical Center, National Institutes of Health.

BRAF and NRAS genotyping

PCR was performed from 50-ng genomic DNA using the HotStarTaq Master Mix Kit (Qiagen, Valencia, CA) and the following primers: BRAF exon 15 forward—5′-TCATAATGCTTGCTCTGATAGGA-3′, BRAF exon 15 reverse—5′-GGCCAAAAATTTAATCAGTGGA-3′; NRAS exon 2 forward—5′-ATAGCATTGCATTCCCTGTG-3′, NRAS exon 2 reverse—5′-CACAAAGATCATCCTTTCAGAGA-3′. PCR products were labeled using a Big Dye terminator kit version 3.1 (Life Technologies, Carlsbad, CA). Sequencing was performed using a 3730 Genetic Analyzer (Life Technologies) and analyzed by the Sequencher software (Gene Codes, Ann Arbor, MI).

Array comparative genome hybridization

All aCGH studies were conducted using Agilent's oligo aCGH platform. Briefly, 1 μg of genomic DNA per sample was directly labeled with a Genomic DNA Enzymatic Labeling Kit, prepared for hybridization with the help of an Oligo aCGH Hybridization Kit, and hybridized to 105K Human Genome CGH 105A Oligo Microarrays. Arrays were washed with Oligo aCGH Wash Buffers and scanned in a High-Resolution Microarray Scanner (all from Agilent, Santa Clara, CA). Data were deposited in the GEO public database under GSE38187.

RNA isolation

Total RNA was isolated using Qiagen's RNEasy Mini Kit, by following the standard protocol.

GX microarray

For expression array studies, the Affymetrix Gene Array System was used. Briefly, 250 ng of total RNA per sample was amplified using a WT expression kit. Next, cDNA was labeled with the help of a GeneChip WT Terminal Label and Control Reactions kit. Samples were then prepared for hybridization using the GeneChip Hyb Wash and Stain Kit and loaded to Human Gene ST 1.0 Arrays. Arrays were washed, phycoerythrin-labeled on a GeneChip Fluidics Station 450, and loaded into a GeneChip Scanner 3000 7G with autoloader for scanning (all from Affymetrix, Santa Clara, CA). Data were submitted to GEO and made publicly available under accession GSE38187.

Microarray data analysis

Agilent aCGH microarray data were imported into the Partek Genomics Suite software (Partek, St Louis, MO), quantile-normalized, and preprocessed using a built-in chromosomal segmentation algorithm (Hawthorn *et al.*, 2010). Individual chromosomal segments were defined as continuous regions covered by at least 10 consecutive microarray probes, a significant ($P<0.001$) and considerable (>0.3 copies on average) difference between the CN of the given segment and neighboring segments, accepting an error rate of $<\pm 0.3$ copies. Segmented genomes were subjected to MDS to describe intersample relationships. Partek's one-way and two-way RM ANOVA analyses were performed on segment CNs to identify CNAs different between individual patients, CNAs consistently changed in consecutive metastases of the same patient, and CNAs between recurrent and random cancer samples. To avoid overestimation of patient-to-patient differences in CNA studies analyzing a mixed-gender group of patients, X and Y chromosome–related data were excluded from all such analyses. Significant differences were identified with a nominal $P<0.05$ and were corrected with FDR of <0.05. Homozygous deletions ($-/-$) were identified as segments with CN<0.4 at an error rate of $<\pm 0.3$ copies.

Affymetrix GX data were imported to Partek Genomic Suite (Partek, St Louis, MO), quantile-normalized, and batch-corrected using distance-weighted discrimination, as described elsewhere (Benito *et al.*, 2004). MDS, one-way, and two-way RM ANOVA analyses were performed as described above. CN and GX data were integrated and analyzed with help of Partek Genomic Suite. Genes whose expression levels were found to be affected by CNAs were identified by computing Pearson's correlation between CN and GX values. A Pearson's correlation of $R>0.3$ with $<P0.05$ and FDR 0.05 was accepted as proof for CNA-affected GX.

Flow cytometry

Cells were harvested nonenzymatically using Cellstripper (Corning, Manassas, VA) and stained with LIVE/DEAD Kit (Life Technologies, Carlsbad), anti-CD117 (KIT)-APC (BD Biosciences, San Jose, CA) or isotype controls. Data analysis was performed using a MACSQuant Analyzer (Miltenyi Biotec, Teterow, Germany) and FlowJo (TreeStar, Ashland, OR).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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